Exploring the benefits of inhibiting HIF and Notch to overcome resistance to cancer therapy

Summary

Cancer is the leading cause of death worldwide according to the World Health Organization (WHO). Despite the major advances in our understanding of cancer biology, efficient diagnosis and improvement of anticancer therapies, the burden of cancer is increasing worldwide. Some tumors initially respond to treatment but ultimately acquire resistance to chemo-radiotherapy which leads to tumor recurrence. Normal cell renewal in adult tissues and adaptation to low oxygen levels are regulated by several signalling pathways that often are deregulated in cancer and contribute to treatment failure. In this thesis, we studied the effects of inhibiting two important signalling pathways in cancer, NOTCH and HIF, to overcome resistance to cancer therapy.

Hypoxia is a major feature in many solid tumors and arises due to an inadequate and immature vascular function resulting in a decreased delivery of oxygen in nutrients. Hypoxia areas within the tumors show resistance towards conventional treatment modalities such as radio-chemotherapy, causing a reduction in survival of cancer patients. One of the most recognized mechanism to adapt and survive hypoxic stress conditions is mediated via stabilization of the hypoxia-inducible factors (HIFs), mainly consisting of HIF-1α and HIF-2α. Under hypoxic conditions, HIFs-α dimerize with HIF-1β, which forms a transcriptionally active dimer that binds to the hypoxia responsive elements (HRE) in the promoter regions of hundreds of genes to enhance to expression of many hypoxia-related genes. Direct target genes of HIFs have been shown to be functionally related with many tumor-associated characteristics such as energy metabolism, angiogenesis, tumor metastasis and resistance to therapy. HIF-1α and HIF-2α show unique but also overlapping roles in regulating many of the hypoxia-related features seen in hypoxic tumors.

One of the main aims of this thesis was to investigate the differential role of HIF-1α and HIF-2α in regulating tumor-associated characteristics in view of the possibility of implementing them in a personalized treatment approach and to use them as independent prognostic factors in cancer. Several clinical studies investigate the prognostic role of HIF-1α in cancer, including many meta-analyses for all types of cancer and different HIF-1α polymorphisms. Prognostic data on HIF-2α can be found for some of the clinical studies included in these meta-analyses, which often differ from HIF-1α data. However, many discrepancies are seen among the investigations that were performed during the last decades, in which HIF-2α has been reported to be either a positive or negative prognostic factor in cancer. To expand the knowledge on the prognostic value of HIF-2α in cancer, **chapter 2** describes a comprehensive overview the prognostic data of patients suffering from diverse types of cancer according to

their intratumoral HIF-2α expression. Here, we demonstrate that HIF-2α expression is a negative prognostic factor when evaluating 5 out of the 6 endpoints tested, including overall survival, disease-free survival, disease-specific survival, metastatic-free survival and progression-free survival. An inverse relationship found when evaluating renal cell carcinomas, in which HIF-2α seems to behave as a positive prognostic factor depending on its subcellular localization. The results of this meta-analysis therefore support that development of a clinically applicable test to assess HIF-2α expression to estimate patient prognosis.

The outcome of this meta-analysis also suggests its use as a target for anti-cancer therapy. Therefore we studied the consequences of genetically depleting both HIF-1α and HIF-2α in radiation sensitivity in cancer cells in **chapter 3**. To directly assess the unique and overlapping functions of HIF-1α and HIF-2α, we use CRISPR gene-editing to generate isogenic H1299 non-small cell lung carcinoma cells lacking HIF-1α or HIF-2α or both HIF1 and HIF2. When cultured under hypoxia HIF-1α-deficient cells strongly upregulate the levels of HIF-2α probably through enhanced protein stabilization or reduction of its degradation. In accordance with our published meta-analysis describing the negative impact of HIF-2α in cancer, we observed that HIF-1α-deficient cells, which overexpress HIF-2α, are more radioresistant than wild type cells when irradiated under low-oxygen conditions (0.2% O_2). A Higher survival of irradiated HIF-1α-deficient H1299 cells was associated with a reduced recruitment or accelerated repair of γ-H2AX foci directly after irradiation and not due to differences in proliferation. HIF-1α-deficient cells were used as a cellular template to target HIF-2α using the CRISPR/CAS9 system, thus generate a double-HIF-1/2α defective cell line and confirm that HIF-2α is responsible for the radioresistant phenotype. On the contrary, HIF-1/2α-deficient cells conferred radiosensitivity to H1299 cells under both normoxic and hypoxic conditions. *Lactate* has been hypothesized to mediate *radioresistance by virtue of its antioxidant properties, mediate resistance to apoptosis and promote a stem cell phenotype. We thereby hypothesize that the increased extracellular lactate levels in* HIF-1α-deficient cells contribute to the induction of a radioresistant phenotype. Furthermore, a strong reduction in basal glycolytic activity was seen in HIF-1/2α-deficient cells possibly contributing to the higher radiation sensitivity phenotype. This suggests that there is an urgent need for better understanding the combined function of the HIF proteins in cancer and further translate it into clinical application in patients.

NOTCH performs activities that have key roles during embryonic development and also in tissue maintenance of adult tissues. NOTCH is also associated with cancer by modulating

resistance to standard treatment modalities such as radiotherapy and chemotherapy. Moreover, Notch is often deregulated in certain types of cancer, which makes it a promising targeting candidate by using γ-secretase inhibitors. However, many side-effects of γ-secretase inhibition were seen which are related to on target Notch toxicity in the intestine causing intestinal goblet cell metaplasia and other tissue-related toxicities. Previously, our group has shown the benefits of combining NOTCH inhibition with radiotherapy in a lung cancer model. No models are currently available to study the effect of radiotherapy and Notch inhibitors on normal tissue response in patients with lung cancer. Therefore, in **chapter 4** we developed a model to investigate the role of the Notch signalling in cell fate decision and the radiobiological properties of primary bronchial epithelial cells (PBECs) isolated from human lungs. In particular, our work has demonstrated that NOTCH signalling plays a crucial role in modulating cell fate decisions by modulating the differentiation and self-renewal capacity of PBECs. The capacity of modulating a specific cellular balance within the lung by blocking NOTCH is further enhanced by the effect of radiation. Based on the results from this chapter it can therefore be concluded that NOTCH inhibition increases the number of lung stem cells in irradiated PBECs cultures by enhancing the DNA damage response and thus reducing the amount of double-strand breaks.

In **chapter 5**, we investigated the activity of the different components of the γ-secretase complex on a mouse embryonic fibroblast (MEF) cellular model expressing wild type of oncogenic NOTCH1. Using this mouse model, we demonstrate that both NOTCH isoforms cleaved by the different components of the γ-secretase complex show characteristic cleavage and activation capacities that lead to a differential gene expression pattern. Our results reveal an unexpected preference of the Psen2:Aph1B γ-secretase complex to process oncogenic mutant NOTCH1 receptors over wild type NOTCH receptors. Psen2-specific targeting could be exploited in cancer cells while sparing Psen1 substrates including Notch signaling in normal cells were Psen2 is non-essential. We also used chloroquine as an inhibitor of the endocytic pathway and observed that oncogenic NOTCH1 protein processed by Psen2:Aph1B preferably relies on the endocytic pathway for its activation and latter function as a transcription factor.

Finally, **chapter 6** summarizes the findings of this thesis which describe some of the benefits and limitations of blocking HIF and NOTCH in cancer.